The University of Chicago

A TOPOGRAPHICAL AND CYTOLOGICAL STUDY OF THE SYMPATHETIC NERVOUS COMPONENTS OF THE SUPRARENAL OF THE CHICK EMBRYO

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

By
ALFRED BRAUER

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A TOPOGRAPHICAL AND CYTOLOGICAL STUDY OF THE SYMPATHETIC NERVOUS COMPONENTS OF THE SUPRARENAL OF THE CHICK EMBRYO [1]

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FIVE TEXT FIGURES AND ONE PLATE (THIRTEEN FIGURES)

AUTHOR'S ABSTRACT

A study was made of the development of the suprarenal gland of the chick with reference to the following points: 1) Topographical relationship of the sympathetic cell masses to the cortical cords, and to the sympathetic nervous system. 2) Cytological differentiation of the sympathetic cells.

The migrating indifferent sympathetic cells, although becoming generally distributed over the medial surface of the cortical ridge, form a series of more prominent aggregations at certain definite loci. These aggregations are the rudiments of two longitudinal ganglionic chains, which develop from the sixth to the seventh day. Around and between the ganglionic rudiments the indifferent cell groups become penetrated by sympathetic fibers from the ganglionic rudiments as well as by fibers of sympathoblasts which develop from indifferent cells of the sympathetic-chromaffine complex. At the same time multipolar sympathetic cells lying between the cortical cords send processes to the tracts developing between the ganglia on the surface of the cortical ridge.

Differentiation of 'chromaffine cells' from indifferent cells of the aggregations begins during the eighth day, after a profuse innervation of the rudiments has been established. Differentiation is evidenced by the appearance of cytoplasmic granules, and by the appearance of the 'chromaffine reaction.' The possible bearing of the innervation of the indifferent cell aggregations upon their differentiation and upon their penetration of the cortical bodies is discussed.

INTRODUCTION

From the extensive literature on the development of the suprarenal gland (Poll, '06), the points of agreement of most of the present investigators are: the gland has a double origin, the cortical portion is derived from a cellular proliferation of the coelomic epithelium, and the medullary portion from cells of the sympathetic nervous system. The latter cells arrive in ultimate relationship to the cortical portion by an active penetration beginning some time after the two elements first come into contact with each other.

[1] This work was done largely at the zoölogical laboratory of the University of Kentucky during 1929 and 1930. It was completed during the author's leave of absence from the University of Kentucky, while in residence at The University of Chicago, 1930 and 1931.

The suprarenal of the chick, with which this paper deals, as that of birds in general, differs from the suprarenal of mammals in that the cortical tissue and the medullary tissue are topographically not arranged as the names *cortex* and *medulla* imply, but are irregularly intermingled in the form of cords. Again, the development of the chick's suprarenal is complicated in that the sympathetic system, from which the medullary tissue takes origin, arises in successive steps. These rather definite stages are called the primary and the secondary sympathetic chains, respectively (His, '97). Rau and Johnson ('23) have shown that the so-called primary chain is but a transitory stage in the development of the true sympathetic chain, and that the elements of the former are incorporated more or less completely into the latter.

The so-called primary chain is by a number of authors considered to be responsible for the contribution of the medullary primordium as well as for the innervation of the glands (Rabl, '91; Fusari, '93; Soulie, '03; Rau and Johnson, '23). Of these, Rabl holds that the migration of elements to the cortical rudiments also continues from the 'secondary sympathetic chain.' Abel ('12) describes a migration from the sympathetic system as late as the seventh day, but, according to her, these cells differentiate into sympathetic nerve cells in this region. Goormaghtigh ('21) has likewise seen a migration of sympathetic cells from the primary sympathetic chain, but, according to his view at that time, the sympathetic cells are intermingled with mesodermal cells which give rise to the medullary tissue, while the former contribute the nervous elements of the suprarenals.

Recently, the problem of the formation of the suprarenal glands in the chick was analyzed experimentally. Willier ('30), by means of the chorio-allantoic grafting method, demonstrated that histologically complete glands could be obtained from isolations of the wolffian body, containing the suprarenal rudiment, made as early as ninety-six hours. He thereby showed that the cells contributed by the primary sympathetic chain are sufficient for the formation of the

medullary cords. The work further suggested that the cells derived from the primary sympathetic chain form a contiguous mass on the surface of the cortical cords, and penetrate between the latter after lying in juxtaposition to them for several days.

In the above-mentioned work, of which this paper is a direct outgrowth, a number of questions were raised relative to the significance of the relationship between the sympathetic system and the suprarenal, and to the differentiation of the medullary tissue of the gland. It is the purpose of the present paper to record the results of a study of the following points: First, the topographical relationships between the sympathetic cell groups and the cortical cords established prior to the differentiation of the medullary tissue. Secondly, the relationships at the time of cellular differentiation between the sympathetic cell groups and the cortical cords on the one hand and the sympathetic cell groups and the sympathetic nervous system on the other hand. Thirdly, the cytological changes which occur in the indifferent sympathetic cell during its differentiation into a medullary cell.

Acknowledgments. For the suggestion of this problem and for helpful criticism, I sincerely thank Dr. B. H. Willier.

MATERIAL AND METHODS

Chicks of from three days' incubation up to three days after hatching were used. The eggs were incubated under optimum conditions. For the earlier stages (three to six days) as well as those of the intermediate stages in which it was desired to study the sympathetic and nervous relationships of the glands, the entire embryo or at least the entire trunk was sectioned. For the cytological studies, only the suprarenals with enough of the surrounding tissue to prevent injury to the glands were dissected out and prepared. In all cases serial sections were made. A total of 160 embryos were prepared.

For general purposes Zenker's, Bouin's, or Tellyesnicky's fluids were used. For sensitive tests of the chrome reaction, Regaud's formol bichromate, with neutral formalin, or Orth's fluid modified (potassium bichromate, 2.5 per cent, 20 parts; neutral formalin, 1 part), were most successful. After this fixation the sections were stained with Ehrlich's hematoxylin, without a counterstain. For the Golgi apparatus, Mann-Kopsch's long osmic-acid method, Cajal's uranium nitrate, or his neutral-formalin methods were employed. These methods likewise served for the demonstration of nerve fibers. The mitochondrial techniques employed were: fixation in Regaud's fluid with neutral formalin, or in Helly's formol-Zenker (neutral formalin) followed by osmication in 2 per cent osmic acid for two to three days, gave the best results. The sections were stained with Bensley's aniline-acid-fuchsin, methyl green. A staining method which was frequently used after Zenker's fluid or Bouin's fluid was Masson's hematoxylin-saffranine-erythrosine. This gave splendid differentiation of the sympathetic tissue as well as of nerve fibers in the gland.

ANATOMICAL RELATIONSHIP OF THE SYMPATHETIC NERVOUS SYSTEM TO THE SUPRARENAL BODIES DURING VARIOUS STAGES OF THEIR DEVELOPMENT

1. Initial relationship of the sympathetic cells and the cortical nodules

Origin of sympathetic cell groups at the suprarenal level. Examination of sections of chick embryos of from ninety to ninety-six hours' incubation shows in the anterior portion of the trunk region, or more specifically, from approximately the tenth to seventeenth somite level, segmentally arranged cell aggregations, more closely massed and more heavily staining than the surrounding mesenchyme, lying on each side, dorsolateral to the aorta. The aggregates on each side are connected by loosely interspersed cells, and thus form a sort of chain. They constitute the so-called 'primary chain' of sympathetic ganglia (His, '97). Trains of cells of similar

appearance, lying just posterior to the segmental aortic branches, extend from the spinal ganglia down to these ganglionic masses, and likewise from the masses ventrally, around each side of the aorta into the dorsal mesentery. At the level of the suprarenals, with which we are more particularly concerned, from the seventeenth to the twenty-third somite, and for several somites beyond, the primary sympathetic ganglia are not so prominent. Here the sympathetic cell trains extend from the spinal ganglia, medioventrally to the level of the aorta, and thence ventrally around the sides of the aorta without forming such large aggregations dorsolateral to the aorta.

Due to the alignment of the cells of the trains, to their continuity, and to their morphological similarity to the cells which first appear in the spinal ganglia, it is assumed that they are cells which have migrated by way of the developing spinal ganglia.

From the ganglionic masses of the primary chain while the cells are still apparently in an indifferent stage, cells extend outward ventrolaterally into the loose mesenchyme between the aorta and the wolffian body. Likewise, cells appear to become detached from the cell trains proceeding ventrally from the sympathetic ganglia, as well as from the cell trains of the primary sympathetic tract between the spinal ganglia and the sympathetic ganglia. Apparently these detached cells or cell grouplets are migrating in a posterior direction, since by the end of the fourth day they are found not only in the sections showing the primary sympathetic nerve tracts and ganglia, but also in successive sections between these ganglia and their tracts. Detached sympathetic cells are found in the mesenchyme between the aorta and the wolffian bodies during the fourth day, where they come in contact with the proliferation of coelomic epithelium which is giving rise to the cortical cords of the suprarenal glands. Wherever these indifferent sympathetic cells come into contact with obstructions, such as blood vessels or organs, they collect. Mechanical influences of this nature appear to play a most important

part in the establishment of the permanent ganglia of the surface of the suprarenal bodies, as will be shown later.

Factors concerned in establishment of the sympathetic-chromaffine rudiment. In sections of appropriate levels, namely, from the posterior portion of the seventeenth- to the twenty-second-somite level, the cortical ingrowths are seen proliferating from the thickened coelomic epithelium, which from the twentieth to twenty-second somites becomes continuous with the germinal epithelium. This proliferation produces a series of nodules extending dorsally into the mesenchyme along the medial surface of the anterior portion of the wolffian body. Here they form a ridge about a millimeter in length, which by late on the fourth day has become detached from the coelomic epithelium.

During the entire fifth day, these cortical ridges, one on each side of the aorta, consist of nodules separated by mesenchyme. Crowded by the developing mesonephroi lateral to them, the further growth of the nodules takes place chiefly in a dorsomedial direction. The result is the formation of cortical cords from the nodules. The extension of these cords gives to this portion of the gland the form which it retains to a considerable degree in the definitive state.

At the time the cortical cells are being proliferated from the coelomic epithelium, the first of the indifferent sympathetic cells (sympathogonia, of Poll), which have pushed into the loose mesenchyme from the primary sympathetic cell trains, come into contact with them. As the cortical nodules become larger, and are detached from the coelomic epithelium, they form a ridge lying medial to the mesonephros on each side of the body. The sympathogonia collect over this ridge. At the anterior portion of the ridge they come in contact with its dorsal and medial surfaces and collect in front of its anterior extremity. Over the remainder of the ridge, many of the sympathetic cells remain in contact with it, while others, which remain in the mesenchyme, form trains continuing ventrally. These indifferent sympathetic cells form the rudiment of the sympathetic-chromaffine complex which develops in association with the cortical cords.

The first and most extensive portion of the sympathetic-chromaffine rudiment is at least partially established by the end of the fifth day. This is the 'anterior cap' of indifferent sympathetic tissue.

Due to the fact that sympathetic cells from the primary sympathetic ganglia and from their tracts become detached and are subsequently found in sections posterior to them, these indifferent cells probably reach their final locations at segment levels posterior to the ones containing the tracts from which they originally came. This makes it impossible to state from which particular primary sympathetic ganglion or tract the sympathetic cells of this anterior cap have come. Thus, although the anterior extremity of the cortical ridge at this time lies in the posterior part of the seventeenth somite, it is evident that the primary sympathetic ganglia of more anterior somite levels have contributed to the sympathetic elements around the cortical nodules.

Another feature which brings the anterior ganglia nearer to the region involved is the extreme curvature of the chick on the fourth day. This causes the sympathetic trunks to converge somewhat like the radii of a circle toward their axis. Spinal ganglia which in a day or two will lie considerably farther anterior, due to the elongation and straightening of the embryo, are thus connected with primary sympathetic ganglia lying quite near the suprarenal rudiment.

By 120 hours the cortical bodies occupy more of the free area between the aorta and the wolffian body. Increasing numbers of the sympathetic cells collect on their medial border. At the anterior end of the cortical ridge it is found that the cell trains extending ventromedially in the sympathetic tracts along the posterior surfaces of the dorsal branches of the aorta, come into contact with the more dense mesenchyme around the aorta, turn laterally, and come into contact with the anterior end of the cortical ridge. Here the trains bifurcate. Most of the sympathogonia turn ventrally along the medial surface of the cortical rudiment and become applied to its irregular border. The remainder turn laterally, around

the anterodorsal surface of the cortical ridge, and here form a sort of cap of indifferent sympathetic cells.

The anterior cap of indifferent sympathetic cells, which is laid down from the fourth to the sixth day, constitutes the rudiment of a ganglion as well as of a considerable portion of the prospective medullary tissue which penetrates the gland at a later time. The cap eventually (by the end of the sixth day) covers approximately the entire anterior third of the corticular rudiment, due to the accumulation of additional cells and to the posterior movement of its indifferent cells.

At about one-third the distance of the length of the gland rudiments, reckoning in a craniocaudal direction, lies the next primary sympathetic tract from which cellular elements extend directly to the gangliomedullary primordium. This is the primary sympathetic tract of the twentieth-somite level. As from the preceding one, cellular elements extend from this tract ventrally around the sides of the aorta. At this level the omphalomesenteric artery leaves the aorta and passes obliquely to the right. The aorta wall near the base of the omphalomesenteric artery is stretched to the right, close to the suprarenal body of this side. The subcardinal vein lies ventral and slightly medial to the suprarenal body. The suprarenal body itself slopes ventromedially and appears to become adjusted to the angle formed by the two blood vessels named and the mesonephros lateral to it. The right gland rudiment now lies almost directly in line with the sympathetic cell trains. The indifferent sympathetic cells collect on its medial surface and between it and the aorta and, on its medioventral border, over the subcardinal vein (fig. 1).

The left suprarenal rudiment at the corresponding level lies outside of the direct line of the sympathetic cell trains. No large blood vessels are present on this side. Relatively few sympathetic cells aggregate over this portion of the gland. Later, this is conspicuous as a real difference between the left and the right glands in their definitive form.

These anatomical features are practically identical in each of six five-day chicks, and in each of ten six-day chicks studied

for this purpose. In two of the former and in four of the latter the position of the right gland is slightly more ventral and more nearly in line with the sympathetic cell trains. In each of these a relatively greater number of indifferent cells have accumulated on the dorsomedial surface. In all of the embryos, however, more cells have accumulated on the anterior, medial third of the right gland than on the corresponding part of the left.

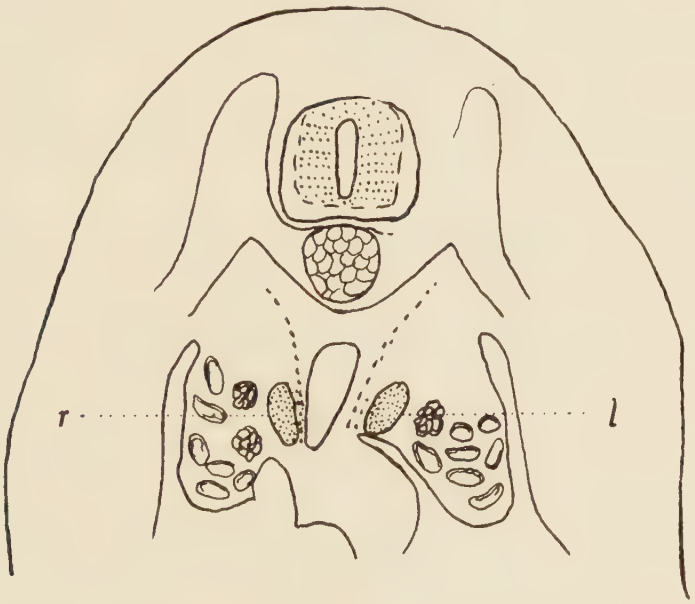

Fig. 1 Section through level of twentieth somite of 123-hour chick. Right suprarenal more directly in path of migrating sympathogonia. *r.*, right side; *l.*, left side.

Midway down the length of the glands the sympathetic ganglia of the twentieth-somite level contribute to the premedullary components. Here the cell trains on each side divide around an arterial branch which leads from the aorta to the mesonephros, and which lies just dorsal to the cortical ridge. The sympathetic cells course around each side of the artery and become distributed over the medial surface of the cortical body just under it.

About four-fifths of the way down the length of the glands an anastomosis between the right and the left subcardinal veins is established on the fifth day. Over and around this anastomosis the indifferent sympathetic cells collect and form a number of aggregations which are the rudiments of the splanchnic plexus ganglia. These rudiments are in close relationship with the posterior third of the suprarenal bodies. At the time of their establishment, early on the sixth day, there is no apparent difference between the cells applied to the surface of the cortical ridge and a majority of those with which they are continuous in the anlagen of the splanchnic plexus ganglia. A few of the cells of these masses, however, are beginning to show indications of differentiation into ganglion cells.

The development of the secondary sympathetic chain of ganglia begins to take place during the fifth day, and the elements of the primary chain are incorporated into it (Rau and Johnson, '23). The ganglia of this chain are located at or around the bases of the sympathetic trunks and medial to the ventral roots of the spinal nerves. The definitive sympathetic trunks become well established during the sixth day. At the level of the suprarenals they extend ventromedially along the posterior surfaces of the segmental aortic branches. They coincide with the primary tracts about three-fourths of the distance to the original primary ganglia, where they diverge laterally, and lead directly to the sympathetic-chromaffine masses on the dorsal surfaces of the cortical glands.

Cells appear along these tracts which in all particulars correspond to the indifferent cells of primary tracts, and to those around the cortical bodies of the suprarenals. These cells appear to be added to the sympathetic-chromaffine rudiments, since there is obviously an increase in the elements of these masses up to the seventh day. By later differentiation, these cells appear to be capable of forming sympathetic nerve cells as well as chromaffine cells.

On the seventh day important developments occur in the sympathetic-chromaffine rudiments around the cortical

glands. These cell masses have accumulated more heavily at three distinct points, at the anterior end, in the center, and at the beginning of the posterior third of the cortical ridges. They correspond not so much to the migration paths of sympathogonia, but more precisely to the position of the large vessels in close relationship to the cortical bodies. Spread between these masses, but not so heavily as at the points mentioned, sympathetic cells also cover the remainder of the medial surfaces. Between the aorta and the suprarenals, somewhat nearer the aorta, are found sympathetic cells which by methylene-blue, Mann-Kopsch osmication, or Masson's triple stain, are seen to have developed nerve fibers connecting them with the sympathetic trunks leading dorsally to the definitive sympathetic ganglia and ventrally around the aorta to the visceral ganglia. At the posterior level of the suprarenals they lead to the large plexus ventral to the aorta.

Developing between the ganglionic masses on the surface of the cortical gland, and connecting them in the form of longitudinal, anteroposterior chains, are the definitive sympathetic trunks of this level. A trunk connects the ganglionic masses on the mediodorsal surface of each cortical body, another connects those on the medioventral edge. The two chains on each cortical body are connected by dorsoventral connections which lead between the ganglionic masses of the mediodorsal and medioventral ganglionic masses (fig. 2).

Between the sympathetic nerve trunks and the suprarenals are the sympathetic cell groups consisting largely of prospective chromaffine cells. Among the latter, however, are cells which have developed extensive neurofibrils, which from this time on make up a prominent feature of the sympathetic masses. Furthermore, these masses are bounded by, and in close relationship with, other groups whose elements have differentiated into ganglion cells and form the rudiments of sympathetic ganglia. Three distinct cell types are found during the seventh day on the surface of the cortical bodies. They are the ganglionic cells of the rudimentary ganglia, extraganglionic nerve cells, and indifferent sympathetic cells.

RELATIONSHIP JUST BEFORE PENETRATION OF PROSPECTIVE MEDULLARY CELLS

Innervation of the cortical glands. As early as the fourth day (ninety-six hours), occasional indifferent sympathetic cells may be seen lying among the cortical nodules. Fusari ('93) also noted this. By 120 hours these cells have devel-

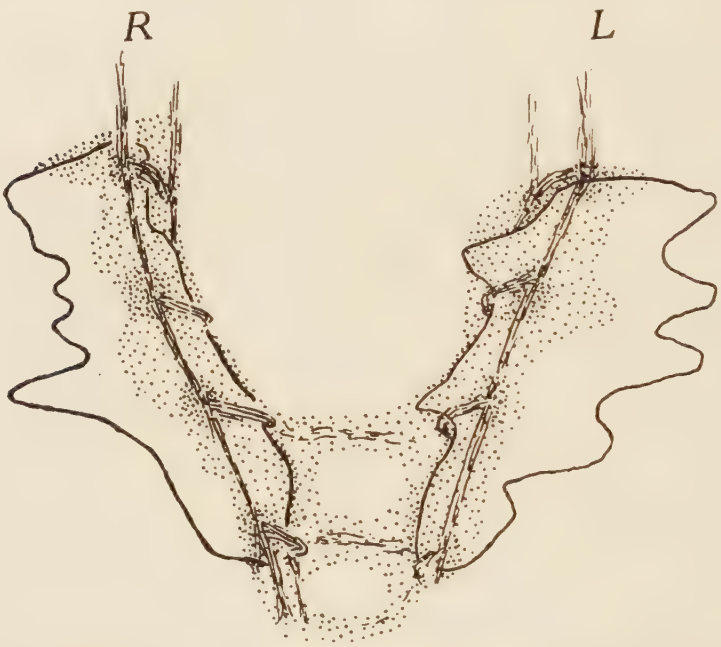

Fig. 2 Reconstruction of chick suprarenals on eighth day, showing distribution of sympathetic components over cortical bodies, and the sympathetic chains on the surface.

oped processes which extend out between the nodules and establish a contact with the processes of other sympathetic cells developing outside of the cortical body, or they may be observed leading directly to the cell masses which are differentiating into sympathetic ganglia on the dorsomedial or ventromedial surfaces of the nodules.

During the fifth day the indifferent sympathetic cells are not invading the cortical mass, since they cannot be observed

in various stages of an actual penetration, as is the case at a later time. On the other hand, there is every indication that the cortical masses have grown up around isolated indifferent sympathetic cells, since such isolated cells are seen in this region at the time that the cortical masses are being proliferated from the coelomic epithelium. At 110 to 120 hours such nerve cells with developing processes can be seen lying in the indentations between cortical nodules, not oriented so as to make it appear that they can be penetrating between the cords (fig. 3). In successive stages, with an extension

Fig. 3 Section at 120 hours' incubation, showing cortical nodules with sympathetic nerve cells differentiating between them.

of the cortical nodules, the nerve cells appear more numerous in the sections and deeper in the cortical bodies. That the cortical nodules, however, are exhibiting a rapid extension is evident by their increasing mass and by the great mitotic activity of their cells.

Late on the sixth day the nerve cells within the cortical glands have extensive processes. Their processes are closely applied to the cortical cords and others lead outward to the ganglionic masses. Six to eight of these relatively large cells may be observed in almost every section throughout the gland. By the seventh day the multipolar nerve cells within

the cortical glands are better developed. By use of osmic-acid agents, their cell bodies are conspicuous, and a veritable reticulum is formed among the cortical cords by their processes. A distinct nervous association is formed between them and the ganglia lying medial to the gland (fig. 4).

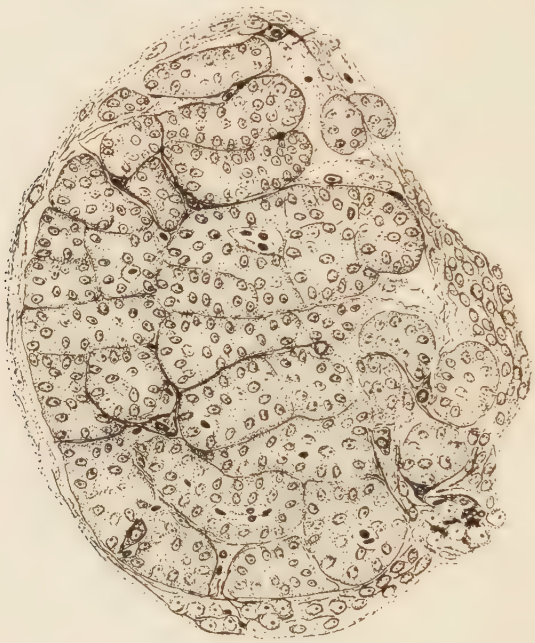

Fig. 4 Section through right suprarenal body early on seventh day, showing multipolar sympathetic cells lying between cortical cords, and the sympathetic-chromaffine complex on the surface of the cortical body.

Innervation of the sympathetic masses. Outside of the cortical body, an extensive nervous network develops during the sixth and seventh days. Making up this network are: 1) Nerve processes which connect the ganglionic masses on the medial surface of the gland, and which form the definitive trunks between the ganglia, and between the two longitudinal chains of ganglia. 2) Nerve processes which lead from the developing ganglia into the groups of indifferent cells. 3) Fibers of sympathoblasts, which differentiate among the

indifferent cells outside of the ganglionic rudiments. The processes of these cells course between groups of indifferent cells and along indifferent cell trains, apparently bringing the indifferent cell groups into association with each other. With the indifferent cells the sympathoblasts make up a sympathetic-chromaffine complex.

From the time that the indifferent cells first collect in the vicinity of the gland to the time at which the chromaffine cells begin their differentiation, there is being established, through the sympathetic cells and their processes, a corticomedullary relationship. This extends from the multipolar cells within the cortical body to the ganglia on the surface of the gland, and from these ganglia to the indifferent cell masses, through the sympathetic network.

The complex is described according to its appearance after the Mann-Kopsch osmic-acid technique, but it is readily demonstrated by the Cajal method or by Masson's triple stain. After the osmic-acid method, the nerve fibers are blackened apparently because a thin layer of myelinated material is being deposited around them. The fibers are not blackened with maximum intensity until the ninth day, but may be followed as early as the seventh day.

RELATIONSHIP DURING PENETRATION OF MEDULLARY CELLS

By the end of the seventh day, nerve fibers passing between the ganglionic masses on the cortical glands are well developed so that these ganglia, and with them the rudiments of the suprarenal bodies, are in connection with the sympathetic trunks. By the middle of the eighth day, which, as we shall see, is the time at which the medullary cells begin to penetrate the cortical glands, the sympathetic system in this region has undergone an extensive development and is nearing its final form. The system at the level of the suprarenals consists of the following parts: first, the secondary or permanent chain of sympathetic ganglia; secondly, a chain of smaller ganglia on the dorsomedial border of the suprarenal, and nerve trunks connecting them; thirdly, a chain of ganglia,

ventrolateral to the aorta and ventromedial to the suprarenals, with connecting trunks, and nerve trunks connecting the dorsal and ventral chains. The ganglia of the ventral chain, toward the posterior end of the suprarenal, are among those of the splanchnic plexus.

The indifferent cell masses, by this time containing many nerve cells and nerve fibers, form a continuous sympathetic-chromaffine mass between the ganglionic groups, around the surface of the cortical bodies.

RELATIONSHIP AT TIME OF HATCHING

The suprarenal glands of the chick at the time of hatching are triangular in outline, about 2 mm. in length, lying at the anteromedial angle of the anterior lobe of the kidneys. The breadth of the gland at the widest point is approximately four-fifths of the length. The thickness of the gland in its center is about one-half the breadth. The broadest part of the gland is directed anteriorly, and its apex posteriorly. The lateral border is irregular and closely applied to the anterior lobe of the kidney. At level 3 (fig. 5), in seven of ten chicks, between ages of eighteen and twenty-two days, the left glands are somewhat flattened dorsoventrally, and this portion of the gland extends farther medially so that a portion of it lies ventral to the aorta. In this respect the gland from which the reconstruction diagrams were made is not quite typical.

Location of the sympathetic ganglia. The sympathetic ganglia regularly found in connection with each of the suprarenals are as follow: the first is an anteromedial ganglion forming a sort of cap over this portion of the gland. At the anterior end the ganglion entirely covers the dorsomedial surface of the gland. In level 1 (fig. 5), the dorsal and the ventral portion of the ganglion are still continuous. Slightly farther posterior, the two parts appear as distinct ganglia. This ganglionic cap over the right gland is larger and more extensive than it is over the left. On the right side the dorsal and the ventral portions of the ganglia extend fully a third of the length of the gland without interruption. On the left

side the ganglion extends no farther posteriorly than the anterior fifth of the gland (level 2, fig. 5). This is probably due to the fact that during the period of cell migration, this gland lies more directly in the path of the sympathogonia, as noted previously.

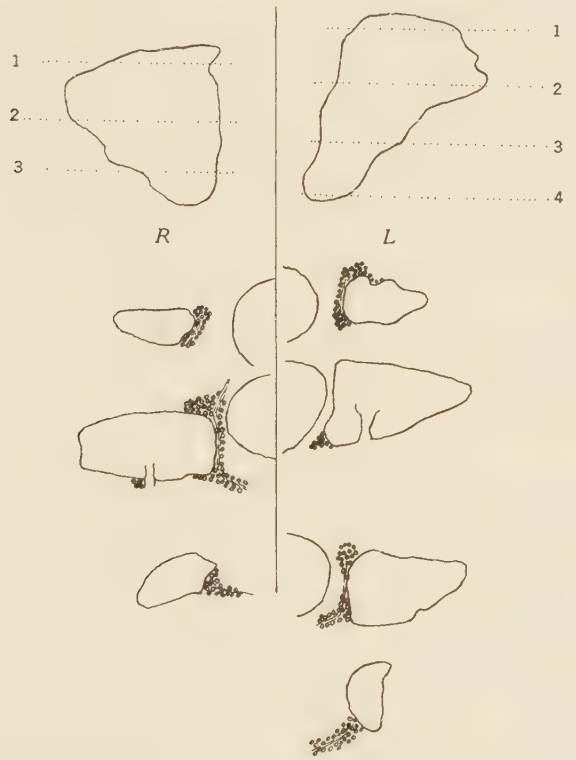

Fig. 5 Reconstruction drawing of suprarenal bodies on eighteenth day of incubation, with transverse sections through various levels showing position of superficial ganglia. *R.*, right; *L.*, left.

A second, ventromedial ganglion begins just posterior to the first, and extends about one-half the remaining length of the gland. The second dorsomedial ganglion begins several sections posterior to the anterior end of the ventral one, and extends several sections posterior to the end of the ventral one.

During the formation of this ganglionic mass, its dorsal and ventral portions were continuous. After the penetration of the cortical gland by the medullary cells, only the ganglionic portions of the mass remained on the surface of the gland, while the intermediate cells penetrated the gland. A sympathetic trunk remained on the surface which connects the dorsal and ventral ganglia of this pair.

Although ganglia at this level of the right and the left glands correspond approximately in position, those of the left gland are not nearly as large as those of the right. This is again correlated with the previous observation that during the migration period, few sympathetic cells collected over the left gland at this level, for the reasons previously given.

A third pair of ganglia, consisting of a dorsomedial and a ventromedial ganglion, is located on the surface of the posterior third of each of the glands (level 3–4, fig. 5). The ventral ganglia of these pairs is continuous with those of the splanchnic plexus, the ganglia of which lie in the midventral line.

There are only minor differences in the position and the extent of the ganglionic masses described in the ten cases studied. In three of these, a small ganglionic mass is found on the ventral side of the gland, just lateral to the suprarenal vein, on the right side.

From the ganglia and from the sympathetic trunks which connect them, many nerve fibers enter the gland. In fact, the inner portion of the connective-tissue capsule, surrounding the glands, is permeated by a rich supply of nerve fibers, and these are seen to penetrate the gland even in sections which show no ganglionic masses.

These observations are based on the study of serial sections of the glands of twelve chicks, between the ages of eighteen days' incubation and three days after hatching. In all of these, the positions of the ganglia correspond.

CYTOLOGICAL STUDY OF DIFFERENTIATING SYMPATHETIC CELLS

1. The different sympathetic cells

The sympathetic cells which first come into intimate relationship with the cortical nodules are called 'indifferent sympathetic cells,' since it is known from their future development that they must have the potencies of developing into sympathetic nerve cells, or into chromaffine cells. From these cells are formed, A) sympathetic ganglia which remain on the surface of the gland; B) sympathetic nerve cells whose fibers innervate the suprarenal gland and its medullary tissue, and, C) the chromaffine tissue of which the greater part enters the gland as medullary tissue, but of which some remains outside of the gland in association with the sympathetic ganglia.

An analysis of the sympathetic cells of the aggregations around the cortical nodules from the ninetieth to the one hundredth hour of incubation, shows a uniformity of structure. The majority of cells are still apparently indifferent as to their future potencies, so far as structural appearances are concerned. By 120 hours, a considerable number have differentiated to such an extent that they can be recognized as extraganglionic nerve cells or as ganglion cells.

The indifferent cells of the primary tracts, and those lying in the loose mesenchyme, which will later come into contact with the developing cortical nodules, differ visibly from the surrounding mesenchymal cells chiefly in two respects; their cytoplasm in general is more extensive, and in its staining reactions more basophilic than that of the mesenchyme cell. This latter difference is sufficient to contrast them sharply against the more lightly staining mesenchyme. The cytoplasm of the indifferent cells may be drawn out in bipolar form, in which case the extended processes are heavier and thicker than the very fine mesenchymal processes of the mesenchyme cells.

Within the cell trains, the cytoplasm of the indifferent cells is indefinite in extent and has an indistinct margin wherever two cells come in contact. They have a tendency

to fuse more or less completely, as though the cytoplasm had a low surface tension. On the other hand, cells are encountered which extend from the cell trains into the loose surrounding mesenchyme, and others are entirely detached from the cell trains.

Measurements of the indifferent sympathetic cells show that the nuclei have an average diameter of $4.2 \times 4.8\,\mu$. The variability from this is slight. The cytoplasm of the cells in the cell trains forms a syncytium in which one cell is continuous with adjacent cells, and consequently cannot be accurately measured. Measurement of isolated sympathetic cells shows that they may be from 8 to $10\,\mu$ from one extremity to the opposite one. The average width is about $4.3\,\mu$. These dimensions remain the same during the fifth and sixth days, until the seventh day, before penetration begins, or up to the time of differentiation of the chromaffine cells, when there is a measurable increase in the cells, the dimensions at that time being $4.3 \times 6.0\,\mu$ for the nuclei, and 5 to $5.6 \times 10\,\mu$ for the cytoplasm.

The indifferent sympathetic cells of the fourth day show a high rate of mitotic activity, approximately one cell in seventeen showing a division phase. This is the highest rate of mitotic activity exhibited by the indifferent cells during the course of development of the gangliomedullary rudiments. During the fourth day, only occasional sympathetic cells are encountered outside of the cell trains and primary sympathetic ganglia. During the fifth day, more and more of these cells accumulate around the aortic branches, and around the cortical cords, but they also show an increase within the cell trains and in the primary sympathetic ganglia. Probably both the addition of cells from without and their high mitotic rate account for the increased numbers. Cell counts on the fifth day, based on five embryos, show that they have a mitotic rate ranging from 1:50 cells, to 1:130 cells. During the sixth day, not nearly so many mitotic figures are seen. Cell counts again based on five embryos showed the mitotic rate to range from 1:242 cells to 1:643 cells. The variability in the rate

of division is too great to warrant any conclusions as to relative mitotic rate.

2. The sympathetic nerve cells

The first visible changes occurring in the indifferent cells of the sympathetic-chromaffin rudiments occur simultaneously in those cells differentiating into sympathetic ganglion cells and in those developing into multipolar sympathetic cells not confined to ganglia, but scattered throughout the sympathetic-chromaffine rudiments and between the cortical cords of the suprarenal. These two cell types may be recognized as early as the sixth day (123 hours), and become quite distinct by the seventh day. The former type of cell in the immediate vicinity of the suprarenal is found to occur chiefly in the loci of the sympathetic-chromaffine rudiments previously designated as centers of aggregation of indifferent cells. During this early period of nervous differentiation (sixth to eighth day), the larger portion of these rudiments is still made up of indifferent cells, but among them sympathetic multipolar cells are differentiating to form a rich nervous network throughout these indifferent cell masses. Within the cortical portion of the gland the indifferent cells which were enveloped by the extension of the cortical cords at this time show the same differentiation.

A. Ganglion cells. During differentiation the nuclei of the indifferent cells show a tendency to become more spherical than the elliptical nuclei which they had as indifferent cells. They appear to be undergoing an enlargement, which, however, does not become conspicuously apparent till the sixth day. The nuclei become clearer and the distribution of the chromatin becomes more uniform. Usually a single large nucleolus becomes more intensely staining and occupies an eccentric position in the nucleus. While the nuclei are undergoing these changes the cytoplasm becomes more basophilic. Cytoplasmic granules become visible around the nucleus. These are especially chromatic to methylene blue. Small grouplets of these cells contrast markedly from the indifferent

sympathetic cells, of the fifth day. By the Golgi methods the rod-like cytoplasmic inclusions around the nucleus become prominent. In addition, a granular, blackened mass usually appears at one end of the nucleus. As the indifferent cell takes the form of a ganglion cell, this mass appears reticular. With the further growth of the cell the reticular body disappears from the polar position and by the fifteenth day lies in the cytoplasm around the nucleus in the form of straight or bent rods. This is the form in which the Golgi apparatus remains in the ganglion cells at hatching (figs. 6, 7, 8).

The mitochondria become most conspicuous in the ganglion cells during the process of differentiation, after these cells have become considerably enlarged (twelfth to fifteenth day). In the indifferent sympathetic cells the mitochondria are small granular bodies lying in the cytoplasm around the nucleus and out in the cytoplasmic extensions. In the ganglion cells they become abundant, though there is a variability in the number in adjacent cells. Here by the Bensley staining method, they are stained bright red. In form they are mostly rod-like, but the rods vary considerably in length. Their orientation in general is parallel to the long axis of the cell, but that this is not necessarily so, is shown by many of the smaller bodies which lie transverse to the longer ones.

From the sixth to the seventh day, by virtue of this differentiation, the ganglia on the dorsomedial and the ventromedial border of the gland become delimited from the mass of indifferent cells around them. By this time the size of the nuclei of the ganglion cells has increased to $8 \times 10\,\mu$ in diameter. The changes which began at from twenty-four to twenty-six hours previously have become accentuated. Fully developed ganglion cells which are found by the fifteenth day may have a nuclear diameter of $12 \times 14\,\mu$.

B. Multipolar sympathetic cells. The cells between the cortical cords, characterized by their large cell body and multiple processes, by the sixth day appear to become more advanced in their differentiation than the developing ganglion cells outside of the gland. By the Mann-Kopsch technique

the nuclei of these cells by the fifth day show an intensely blackened nuclear membrane. Fine granules appear just inside of this, while in the cytoplasm around the nucleus lie coarse blackened bodies. At one end of the elliptical nucleus is a special mass of blackened granules and filaments, identified as the Golgi material (fig. 9). By the sixth day, the nuclei of these multipolar cells stain brown by the osmic technique. Small vesicles containing black granules have been observed on the nuclear membrane projecting outward into the cytoplasm. The cytoplasmic inclusions have greatly increased by this time (fig. 10). The cell featured in this figure was $8.2 \times 10\ \mu$ in diameter.

The Golgi apparatus as in the ganglion cells appears filar and lies farther out in the cytoplasm than it did in the indifferent cells. There is a gradual loss of the Golgi material from the poles of the nucleus and instead it appears in rod-like form in the cytoplasm around the nucleus. In the fully formed ganglion cells the Golgi material appears in the form of twisted rods lying in the cytoplasm, similar to that of the ganglion cell.

3. The chromaffine-medullary cells

Penetration of the gland by the sympathetic-chromaffine complex. The development of the suprarenal from the sixth to the eighth day has been shown to be characterized by an innervation of the cortical cords and of the sympathetic cell masses. The development beginning with the eighth day is characterized by the penetration of the cortical gland by the sympathetic-medullary tissue and the differentiation of the chromaffine cells.

The first indication of penetration appears in embryos of about seven days ten hours (thirty-six embryos). It is first evidenced by the projection between the cortical cords, by strands or trains of cells originating from the sympathetic-medullary complex on the surface of the gland and lying in immediate contact with the gland, extending well in, toward the center. These trains are composed of from five to ten cell

elements. That they are destined to be medullary cells is evidenced by the appearance in some of them of the first positive chromaffine reaction.

When this penetration first becomes apparent, it appears to be due to an amoeboid movement of the cells, in which a single long projection is extended by the cell. The cells are oriented into the intercordal passages. This appears to be similar to the manner by which these same cells arrived in the vicinity of the suprarenal on the fourth day. By the ninth day, this apparent movement of the cells, which can here be observed only by the study of successive stages of penetration, appears to have resolved itself into a general mass movement of the premedullary cells (fig. 11). By 196 hours, large masses of these cells are wedged into the intercordal spaces at the periphery of the gland. At this time all of the cells composing such masses are not oriented in the direction of the flow, as is obvious by the various positions of the elongated cells. Many of these lie transverse to the direction of penetration and appear to be carried along with the general movement of the mass.

Among the penetrating medullary cells the numerous sympathetic nerve cells whose processes ramify among the medullary cells and which help to make up the sympathetic-chromaffine complex also appear to be carried into the gland by the general movement of the cell mass. The nerve cells entering the gland at this time constitute the second group of nerve cells which enter the suprarenal gland, the first having entered as indifferent cells on the fifth and sixth days, while the cortical nodules were being proliferated.

Although the main source of the medullary cells is from the indifferent cell aggregations of the anterior dorsomedial, the central dorso- and ventromedial, and the posterior medial aggregations, the medullary cells do not enter the gland from these loci only, but from the indifferent cells on the surface of the gland between these cell masses as well. Cell trains are seen to extend into the gland from anywhere on the dorsal and medial faces, and from the surface of the gland, where

the indifferent cells were wedged between the cortical cords and the venae cavae. Many of the larger indentations on the surface of the cortical gland contained indifferent cell groups, all of which become centers of penetration.

Penetration appears to begin simultaneously from all parts of the gland surface. It is no more advanced on the dorsal periphery than it is on the medioventral one. Likewise, the strands of medullary cells which extend inward from the ventral side are equally far advanced. The duration of the penetration process is relatively difficult to ascertain, since the medullary cords extend to the surface of the gland even in the definitive condition, and medullary-chromaffine tissue remains around its surface. The definitive condition in the suprarenal appears to be reached, however, by the fifteenth day. By this time the medullary tissue extends throughout the gland, its relative abundance on the surface from this time does not appear to be further diminished. At this time the connective-tissue capsule around the gland also develops. This capsule encloses the ganglia on the surface along with the sympathetic-chromaffine tissue. On the other hand, it excludes some sympathetic-chromaffine tissue from the glands. This remains associated with the splanchnic plexus, and is still abundant here three days after hatching. As late as the thirteenth day, there are comparatively large groups of sympathetic-chromaffine cells at the dorsomedial and the ventromedial borders of the gland, which appear to be reduced considerably by the fifteenth day, while the relative amount of medullary tissue between the cortical cords appears to be increased.

Cytology during transformation. In the differentiation of the indifferent sympathetic cells into chromaffine-medullary cells, several changes occur in the former cells which are probably the result of a single continuous process, the climax of which is the capacity of the cell contents to reduce chromates, or, the 'chromaffine reaction.' This has been shown to be an indication of the presence of adrenaline in the cells (Kingsbury, '11; Ogata and Ogata, '17). The changes taking

place in the cells may be listed as follows: 1) Accumulation of cytoplasmic granules; 2) chromaffine reaction; 3) increase in size of the cells. The succession of events will be described from a single microscopic field in the indifferent cell masses on the surface of the cortical cords, in which various stages of cytological differentiation appear at one time, from the eighth to the tenth day. The differences are identical in each of the preparations made from thirty-two embryos of the eighth day, and thirteen of the ninth day.

Early on the eighth day (174 hours), in the cytoplasm immediately around the nucleus of the indifferent cells, and then in the more peripheral parts of the cytoplasm, faint granules begin to accumulate. These are at first very fine, but soon increase in size and in relative numbers. After fixation in potassium bichromate-neutral formalin, they stain but faintly with the basic stain. After fixation in Bouin's fluid followed by iron hematoxylin, they are more basophilic. After potassium bichromate-neutral formalin fixation, the granules show the faintest trace of a brown shade as early as 174 hours (7J, 7K, 7L). This is, however, too faint and too uncertain to designate as a positive chrome reaction. The granules do not appear to be mitochondrial in nature, since by mitochondrial methods both cytoplasmic granules and mitochondria are visible and show different staining reactions. These cytoplasmic changes are general in the indifferent cells of the medullary rudiment on the surface of the cortical bodies in that they occur simultaneously in different portions of the rudiment; however, not simultaneously in every indifferent cell. The cells showing cytoplasmic granules extend from several sections anterior to the cortical cords (about 70 μ) into the indifferent elements of the splanchnic plexus of the posterior third of the cortical bodies. Identical changes occur in the indifferent cells whether they are in immediate contact with the cortical cords or at a distance of 150 μ or more medial to them, and at a similar distance posterior to their extremities.

The chromaffine reaction. The brown reaction resulting from the union of chromates and adrenaline has been called 'chromaffine reaction' (Henle, '65). The cells showing the reaction were called chromaffine cells (Kohn, '98), 'chromophile cells' (Stilling, '98), or 'pheochrome cells' (Poll, '06). Kingsbury ('11) and Ogata and Ogata ('17) demonstrated that this reaction in the medullary cells is the result of the reduction of chromates by adrenaline, and that the resulting precipitate was chromium dioxide. They demonstrated that the brown reaction in the medullary cell, after treatment with chromates, indicates the presence of adrenaline, and that the intensity of the reaction may be used as an indication of the amount of adrenaline present. The test has since been used by Weyman ('22) and by Miller ('26) to determine the beginning of adrenaline secretion by the medullary cells in the suprarenals of mammalian embryos, and by Okuda ('28) for chick embryos. In the present paper the reaction is likewise taken as an index of adrenaline secretion by the chromaffine-medullary cells and of the completed stage of differentiation of this tissue.

Toward the middle of the eighth day (180 hours), a faint but distinct and definite brown appearance becomes recognizable in many of the elliptical cells of the medullary rudiments while they are still on the surface of the cortical bodies, and in many of the sympathetic cells farther removed from the glands, namely, 150 μ medioventral from the gland, and the same distance anterior and posterior to it. At the level of the posterior third of the gland, such cells showing the same degree of differentiation have been seen in the root of the dorsal mesentery and at the base of the gonads. The reaction makes its appearance simultaneously, in so far as can be determined, in cells of all parts of the medullary rudiment. The picture which one obtains by examining an entire section of the suprarenal rudiment at this time is that of certain cells scattered here and there throughout the chromaffine-medullary rudiment showing a brown cytoplasm. When more closely examined, however, it is found that stages represent-

ing various phases of differentiation are found in the same microscopic field. These stages range from indifferent sympathetic cells, through cells with cytoplasmic granules of several degrees of accumulation, to cells which show the brown reaction distinctly. Among the latter, there are again several degrees of intensity even at this early period of differentiation.

In the appearance of the chrome reaction, as in the appearance of the cytoplasmic granules, the color first becomes visible around the nucleus, in the region of the heavier granules, and then spreads toward the periphery of the cell. This is usually indicated by a more intense reaction in the granular region about the nucleus. The spread of the reaction throughout the cell is rapid, however, as only a few cells can be found in a section, in which all parts of the cytoplasm do not exhibit the same intensity of reaction.

A definite positive chrome reaction in the suprarenal may be obtained after 180 hours' incubation, by the use of Orth's or Regaud's fluids, modified (see Methods). Each of eight cases after the former fluid and each of two after use of the latter gave positive reactions. At 175 hours, the reaction is too faint to be designated as positive, although cytoplasmic granules are present in many of the chromaffine-medullary cells. Glands fixed in Zenker's, Zenker-formol, or Tellyesnicky's fluids, although showing cytoplasmic granules in each of a number of attempts, first showed a definite chrome reaction after ten days. Kingsbury, as well as the Ogatas, noted that both acids and mercury bichloride used with potassium bichromate interfered with the chrome reaction.

By the time the chrome reaction becomes unmistakenly apparent, the penetration of the cortical gland by the chromaffine-medullary tissue has begun. Some of the trains of cells which extend inward between the cortical cords show the reaction, while in others it is not apparent, although these particular trains may extend into the gland as far as those in which it is visible. The cells lying adjacent to or between the cortical cords generally show the brown reaction no more intensely than do those lying 50 to 100 μ from the cords.

All of the elements of a train of differentiating chromaffine cells invariably show the same intensity of the chrome reaction, or the same degree of differentiation during the early early stages. This is quite apparent in the relatively long trains between the suprarenals and the aorta. Trains of from twelve to fifteen elements all in identical stages of differentiation are thus frequently encountered. This is likewise true of the trains extending into the gland. Again, groups of five to ten cells, whose cytoplasm is continuous, are encountered, all of which show identical shades of the brown reaction. When these cell trains and cell groups are analyzed, it is found that the processes of sympathetic nerve cells course along at the sides of the train or through the cell grouplets, forming intimate contacts with them. A train of cells was observed along which the nerve fiber did not extend the entire distance. The train did not come into contact with any other chromaffine cell groups in the section, and was several layers removed from the nearest cortical cord. In this train the cells in the center had a pronounced brown color, while those at each end of the train, which were not in contact with the fiber, showed a gradient of brown reaction diminishing toward the free ends of the terminal cells (figs. 12, 13, 14).

Frequent instances are observed in which remotely separated groups of cells all show a pronounced brown color of the same intensity, and these groups of cells are all connected by the same chain of sympathetic fibers (fig. 13). This is most apparent on the eighth and ninth days, when relatively few cells show the chrome reaction, or at the time of their early differentiation.

By the middle of the ninth day (202 hours), an increasing number of the cells of the chromaffine-medullary rudiment have acquired the capacity to reduce potassium bichromate, and the brown reaction has become more intense in others, presumably those which were the first to acquire it. During the succeeding three days this differentiation continues to a marked extent. This is during the period when the sympa-

thetic-chromaffine tissue is invading the suprarenal glands. Cells which are apparently differentiating into chromaffine cells may be found in groups on the surface of the suprarenal until approximately the eighteenth day. It is doubtful, however, whether any strictly indifferent cells are found later than the fifteenth day. By this time all of the cells associated with the chromaffine rudiments are either sympathetic nerve cells or they show granular cytoplasm characteristic of differentiating chromaffine cells. In the medullary cords, however, cells or groups of cells are always found which show the chromaffine reaction to a lesser degree than others, and some which are almost devoid of brown color. These are probably cells or cell groups in different phases of their secretion cycle.

At the time when the chromaffine cells begin their differentiation the ganglionic cells in the ganglionic areas surrounded by the chromaffine-medullary primordium show an advanced stage of development. The ganglia, confined to the definite regions about the gland which were previously described, continue to become more sharply delimited from the eighth to the twelfth days. This is the period in which the chromaffine cells, too, are undergoing their most marked differentiation. During this period it has never been observed that a ganglionic cell gave rise to a chromaffine cell. The differentiation of each cell type is entirely from the indifferent cells which in their early stages (fourth to sixth days) may give rise to either ganglionic cells, or remain indifferent till the eighth day, and become chromaffine cells. Among the latter, however, cells are frequently found which have well-developed processes resembling neuro processes. These were observed by Remak ('47) and by Mitsukuri ('82). The differentiation of sympathetic nerve cells has been described. These likewise are differentiated from the indifferent cells before the chromaffine cells differentiate.

Size increase of chromaffine cells during differentiation. Accompanying the cytological differentiation of the chromaffine cells from indifferent sympathetic cells, there is a gradual

increase in size which in the embryo attains its maximum in chicks just before hatching. Measurement of a large number of indifferent cells of the sixth and seventh days of incubation showed them to be 8.5 × 5 μ. The nuclei are 5 × 4.3 μ. There is no change in their size during this time. At the time the cells show the first secretion granules, just before the appearance of the chrome reaction, they show a slight increase in size, both in nuclear dimensions and in cytoplasmic measurements. Average cytoplasmic dimensions are 10 × 5.6 μ. Nuclear measurements are 6.2 × 5.3 μ.

Little variation is found in cell size of the chromaffine cells during the period between the eighth and twelfth days. Likewise, the variation in size between the smallest and the largest chromaffine cells is small. Most of them conform closely to the measurements given. By the twentieth day many of the chromaffine cells have dimensions of 10 × 10 μ, or of 14 × 8 μ, while the size of the nucleus has not kept pace with the increase in cytoplasm. Nuclear size is only 5.0 × 3 μ, or 6.0 × 3.5 μ. Many of the chromaffine cells are binucleate. In these the nuclei are small and spherical, measuring but 3.0 × 3.0 μ. Some of the cells with two nuclei probably divide secondarily, as cells are found with but a single small nucleus of these dimensions and appearances. The binucleate cells result from a nuclear fission, or irregular mitotic division, which is noticeable at early stages of differentiation.

The shape of the medullary cells varies widely. It is apparently modified by the positions which the cells occupy in the medullary cords. Some of the cells become closely applied to the cortical cords and become crescent-shaped. Others remain long and spindle-shaped as they were during early stages of differentiation. Still others are oval. After they become chromaffine cells, their cell boundaries become more definite and distinct than they were in the indifferent stage or in early stages of differentiation.

Golgi apparatus of chromaffine-medullary cells. The suggestion that the Golgi apparatus might afford differences in the premedullary cells by which they could be distinguished

at an early time offered itself. Further, the fact that the Golgi apparatus has been demonstrated to occupy a more or less definite position, in the more active region of secreting cells, suggested that it might be taken as an index of cell polarity during penetration of the cortical gland by the chromaffine-medullary cells. Rau and Ludford ('25) had, however, demonstrated that the Golgi apparatus underwent little change in form during the differentiation of chromaffine cells, but underwent considerable change in the differentiation of nerve cells from the same type of cell from which the chromaffine cells originate. Pilat ('12) showed that the medullary cells occupied no fixed polarity with regard to any fixed structures in the suprarenal glands of porcupines, with the position of the Golgi body taken as an index of the cell's polarity.

The Golgi apparatus in the cells of sympathetic trains of the fifth day, and in sympathetic cells around the cortical nodules, consists of more or less scattered material lying in the cytoplasm close about the nucleus, and a more dense mass of blackened material localized at one nuclear pole. The mass appears to consist of an aggregation of granules. The characteristic network is not evident in these early stages. By the seventh day, the Golgi apparatus of the chromaffine-medullary cells beginning to penetrate the suprarenal appears most frequently in a more definitely organized network, lying close to the nucleus at one pole. The transition to this form from that of the earlier sympathetic cells appears to be a spreading out of the blackened material along definite lines in the cytoplasm, so that it appears in the form of rather closely wound loops. Frequently it covers a small portion of the nucleus. In the cells of the trains extending into the gland, the apparatus is found at the anterior pole of these cells (figs. 15, 16). By the eighth day, when the mass movement of the chromaffine-medullary cells into the cortical gland begins, many cells appear to be carried into the gland with the general movement. Here the position of the Golgi apparatus is still a polar one within the cell, but the pole con-

taining the apparatus is not necessarily foremost with reference to the line of penetration. The pole of the cell containing the apparatus during penetration may be directed away from the gland.

In the suprarenal glands of chicks of twelve to fifteen days, in which the invading chromaffine cells have become stationary, the apparatus retains its position at one pole of the nucleus. Adjacent cells within a cell group, however, are oriented in different directions. There does not appear to be a group polarity, but the polarity of each cell appears independent of the adjacent cells. The cells are not oriented in any definite manner with respect to the cortical cords. In cells in contact with the cortical cords, the apparatus is sometimes directed toward the latter, away from it, or so as to indicate a lateral position of the cell with respect to the cortical cord. The position of the apparatus within the cell is fairly constant, but the orientation of the latter has no relationship to the cortical cords (fig. 17).

The Golgi apparatus does not show quite the same form in all of the chromaffine cells. There are gradations ranging from a granular mass in which no vacuoles or vesicles appear, through a more organized arrangement where the blackened material has the form of a closely wound skein in which smaller and larger vesicles appear. These differences appear both after Mann-Kopsch and after the Cajal methods of preparation, so that it may not be altogether due to the blackening of various elements of the cell. The changes are suggestive of physiological phases of the secretion process evidently taking place in the cells.

Mitochondria of the chromaffine-medullary cells. There is little to be found in the literature regarding the mitochondria of the medullary cells. Mulon ('10) described three types of mitochondria in the medullary cells of rabbits and of guinea-pigs. These types were: first, rather long flexible rods occupying a juxtanuclear position and others of the same type interwoven in the center of the cell; secondly, rodlets shorter than those of the first type, and more scattered in the cyto-

plasm; thirdly, granular mitochondria, scattered in the cytoplasm. He regarded these types as transitional between the first and third types, and the latter as giving rise to the chromaffine granules. DaCosta ('13) does not agree with Mulon regarding the shape and distribution of the mitochondria, from a study based on a number of animals. He finds only the granular type, and apparently finds them less numerous than did Mulon. According to DaCosta, they occupy a peripheral position in the cell.

In this work, the mitochondria of the indifferent sympathetic cells are found to be of the small granular type. These bodies are slightly elongate and lie parallel to the long axis of the cell. They are in rather close apposition to the nucleus in these cells, excepting at either end of the nucleus, where the cytoplasm is more abundant. Here they extend out into the cytoplasm away from the nucleus. They occupy this position during the penetration of the cells into the gland (fig. 18). As differentiation of the cells into chromaffine cells progresses, and as the cytoplasm becomes more abundant, the mitochondria are less numerous immediately around the nucleus, but instead, are found to lie more peripheral in the cytoplasm (fig. 19). Stained with acid fuchsin, they are not likely to be confused with chromaffine granules, as is the case after staining with iron-hematoxylin. At the time of hatching they are best seen in the chromaffine cells with a clearer cytoplasm and fewer granules, or in the cells regarded as having emptied their secretion. The nuclei of the chromaffine cells, by the mitochondrial staining methods, may either differentiate as clear vesicles, in which few chromatin granules are distinguishable, or as solidly stained bodies which do not differentiate. DaCosta described these types for the medullary cells of birds and mammals.

DISCUSSION

Developmental mechanics of relationship between sympathetic cells and cortical cords

These relationships owe their origin to the aggregation of indifferent sympathetic cells on the dorsal and medial surfaces of the cortical nodules. The establishment of the aggregations in this position appears to be largely circumstantial, determined, first, by migration of indifferent sympathetic cells along the surfaces of blood vessels to the site of the cortical nodules, and, secondly, to impediments which the migrating cells encounter.

The migration of the indifferent sympathetic cells ventromedially along the posterior surface of the dorsal segmental arteries takes place at all somite levels. Arriving at the dorsolateral surface of the aorta, the cells encounter their first obstruction and form a series of aggregations, which are the primary sympathetic ganglia. From these aggregations cells continue ventrally. Anterior to the cortical nodules, where no obstructions are encountered lateral to the aorta, they continue ventrally through the dorsal mesentery without forming other aggregations. At the suprarenal level, on the other hand, where the cortical nodules become obstructions in the path of the cells, aggregations are formed on the surface of these bodies.

The location of the aggregations on the surface of the gland is again determined by two major factors which are: A) The position and form of the gland, and, B) the position of blood vessels in this level.

At the anterior extremity the conditions are the same in both cortical bodies. Sympathetic cells pushing out from the cell trains are apparently obstructed by the bodies and collect in front of them. The anterior cap of indifferent cells which is thus formed is identical in each of the bodies. Slightly farther posterior, where the aorta wall is stretched toward the right, near the base of the omphalomesenteric artery, the sympathetic cell trains extending ventrally from the primary sympathetic ganglia, come into closer contact with the right

cortical ridge than with the left. The consequence is that more sympathetic cells aggregate on the right gland at the level of the omphalomesenteric artery. At the same level on the left gland, only a small arterial branch appears as an obstruction, over which relatively few sympathetic cells aggregate. The result is a permanent difference in the size of these ganglia in the definitive organs at this level.

Over the posterior third of their medial borders, the sympathetic cells aggregate over the subcardinal veins and establish the rudiments of permanent ganglia in contact with the ventromedial border of each cortical body.

The effect of the form of the gland on the establishment of permanent ganglia is expressed at the twentieth-somite level of the right body, where sympathetic cells aggregate over the flattened dorsomedial border, and become the rudiment of a relatively large ganglion. In the same level, the left gland is not flattened dorsoventrally, and retains few of the sympathetic cells which come in contact with it. Farther posterior, however, the left gland projects medially under the aorta and has a gradual slope. Here large masses of sympathetic cells are retained on its surface.

In addition to the factors named, it is probable that thigmotaxis, by which the indifferent cells tend to follow surfaces, plays a part in the distribution of these cells over the surfaces of the cortical ridges. In the first place, the sympathetic cells arrive at the site of the suprarenals by following the surfaces of the segmental arteries. Then, again, they follow the surface of the cortical ridges, filling indentations, and remaining in depressions and angles. Many push away from the original aggregations and become rather evenly distributed and more closely applied to the surface of the cortical bodies between the aggregations.

The importance of blood vessels and such mechanical factors as flexures have long been recognized as important in the establishment of ganglionic rudiments. Wm. His, Jr. ('93, '97), emphasized their importance in the location of cervical ganglia, where the numerous flexures form mechani-

cal obstructions in the path of migrating sympathetic cells, and in the abdominal region where the veins of the post-cardinal system obstruct these cells. Fusari ('93) noted that the ganglia of the solar plexus formed around the anastomosis of the cardinal veins. He did not point out the influence of the various vessels in the localization of each of the ganglia around the cortical rudiments, although he described the early relationship of the sympathetic elements to the cortical rudiments. Wiesel ('00) regarded the early relationship between the sympathetic cells and the cortical rudiments in the pig as circumstantial, although he did not point out the particular influence determining the localization of individual ganglia.

Innervation of the suprarenal rudiments

The first sympathetic cells to be found among the cortical nodules may be recognized as early as ninety-six hours. Fusari ('93) observed these, and regarded the penetration of the sympathetic cells into the cortical rudiments as a continuous process beginning at this time. He did not recognize the real active penetration which begins on the eighth day. Goormaghtigh ('21) described a double penetration of the gland. The first of these, beginning at four days sixteen hours, he considered to be responsible for the innervation of the gland. Harris ('28) likewise saw nerve cells in the gland at 144 hours, and assumed that they had penetrated previous to the sixth day, and that they were responsible for the innervation of the gland. The degree of differentiation attained by these cells prior to the penetration of the chromaffine cells was not described by these authors.

This 'penetration,' as described in the present paper, is seen to be a penetration not in the sense of an active movement into the gland by the indifferent cells, but is due more directly to the proliferation and the extension of the cortical cords around certain cells of sympathetic origin, found in contact with the cords. Goormaghtigh was partially correct in regarding them as providing the innervation of the gland;

however, they are not responsible for the entire innervation of the suprarenal, since large numbers of nerve cells are later carried into it by the invasion of the sympathetic-chromaffine complex. The first sympathetic cells to differentiate among the cortical bodies before penetration of the gland by the sympathetic-chromaffine complex are a part of the extensive nervous reticulum which is likewise developing in the sympathetic-chromaffine masses.

The profuse innervation of the cortical tissue as well as of the prospective chromaffine tissue prior to the differentiation of the latter has not been stressed. Various authors have emphasized the abundant nerve endings in the medullary tissue of the suprarenals of various animals in their definitive form, but not in their rudimentary form. Remak, as early as 1847, recognized an embryological relationship between the glands and the sympathetic system. Mitsukuri ('82) likewise noted the abundant nerve supply developed in the chromaffine tissue in the suprarenal gland of rabbit embryos, and traced nerves from the medullary tissue within the gland to the ganglia on the surface of the gland. He found the medullary tissue to be derived from the sympathetic cells. Whitehead ('03) described a "chain of ganglia on the dorsal surface of the adrenal of the pig, and another along its ventral surface," similar to those described in the present paper for the chick suprarenal. He did not regard the nerve trunks as having any significance to the gland itself, and stated that there was no reason to regard them as anything but nerves to adjacent anlages. Abel ('12), however, noted a rich nervous development between the aorta and the cortical body in the chick during the seventh day, as well as delicate outgrowths extending between the groups of sympathetic cells. Very recently, Pankratz ('31), considering a possible correlation between the beginning of foetal movements and adrenaline secretion in the white rat, observed a rich nervous development in the prechromaffine tissue, and designated the masses as a 'sympathochromaffine complex.'

According to the present paper, there is a profuse innervation of the sympathetic-chromaffine masses around the cortical rudiments, on the seventh day. During this time the definitive sympathetic trunks become well established in this region and many small nerve cells extend their fibers through the indifferent cell masses, until no groups of cells remain isolated. Furthermore, the nervous network which develops among the cortical cords is continuous with the reticulum which develops outside of the cortical bodies. This network attains its maximum development by the end of the seventh day, several hours before the chromaffine cells show any indications of differentiation. This sympathetic mechanism appears, morphologically, to be capable of integrating the entire complex.

Differentiation and penetration of the chromaffine-medullary cells

The following questions now arise: Is it not possible that, under stimulation through the splanchnic nerves, processes are set up in the indifferent cells which are climaxed by the secretion of adrenaline? Moreover, since the process of differentiation begins at approximately the time that penetration of the gland by a portion of the sympathetic-chromaffine complex begins, and since the chromaffine differentiation occurs in the general vicinity of the suprarenal gland, might not the original stimulus responsible for differentiation originate in the cortical portion of the gland?

The fact that distantly isolated strands or groups of cells, which, however, have a direct nervous connection with each other, through the sympathetic fibers, show an equal degree of differentiation, is striking. Although not all of the prechromaffine cells differentiate into chromaffine cells at the same time, it appears that differentiation of the cells from indifferent cells is inaugurated over a rather short period of time. After the twelfth day, cells in the sympathetic-chromaffine aggregations, which do not show a distinct brown reaction, are nevertheless granular, and apparently in the

process of secretion. They no longer appear like the indifferent sympathetic cells of the early seventh day.

If adrenaline secretion is initiated by nervous stimulation, then the origin of this function appears to resemble the physiology of its secretion in the adult stage, since it is well known that stimulation of the splanchnic nerves increases the adrenaline output of the gland, and that adrenaline secretion, in general, is under the control of the sympathetic nervous system.

Van Campenhout ('27) described a relationship between sympathetic fibers and the primary insular cells of the pancreas of various mammals, similar to the sympathetic-chromaffine complex described in this paper. It was shown by him that the first insular cells to differentiate were always in contact with sympathetic fibers which developed from sympathoblasts in the vicinity. More recently, however, the same author (Van Campenhout, '30) emphasized a differentiation of chromaffine cells in amphibia as taking place before the sympathetics have developed sufficiently to play a part in this differentiation.

It is apparent that the differentiation of the chromaffine cells is not due to immediate contact of prechromaffine and cortical elements, nor to proximity of individual indifferent cells to the cortical cords. The only connection between these two tissues is through the sympathetic network. If the cortical cells do offer a stimulus, the network must be the means of conducting it, since this is the only connection between the two issues. Even so, it might be expected that the indifferent cells bordering the cortical cords, or those lying between them, would be more favorably situated, and would show the reaction earlier and with greater intensity than the more remote cells. It has, however, been shown that this is not the case. This fact has also been emphasized by Miller ('26) for the mouse.

Miller (p. 272) explains the differentiation of the chromaffine cells "in an apparently homogeneous group," on the assumption of "something inherent in the cell, rather than to

its environmental conditions as the cause of this specialization." This statement is interpreted to mean that certain sympathetic cells have the specific value of chromaffine cells prior to their differentiation. This specific value might be due either to qualitative differences in the hereditary mechanism or to cytoplasmic differences in the cells. The first of these explanations is untenable according to the modern view of the cause of cellular differentiation. The alternative one merely presupposes a determination at an earlier time in the ontogenetic history, but, nevertheless, it must still depend upon environmental factors, acting upon a definite hereditary complement.

The sympathetic-chromaffine complex described in this paper is a mechanism which affords sufficient means for the expression of environmental effects upon indifferent sympathetic cells, namely, stimulation through the sympathetic nervous system. Yet, the possibility that the activity of the cortical cells offers such a stimulus, in view of the nervous relationships, is not eliminated. The findings of Willier ('30) are especially suggestive of such a possibility. In chorioallantoic grafts he observed an especially close relationship between the medullary cells and the cortical cords. It would be interesting to note whether the nervous development in such grafts approaches that of the normal.

Concerning the 'delayed penetration' of the chromaffine cells between the cortical cords, first described by Soulie ('03), the following questions arise: Why do sympathetic-chromaffine cells which have lain in juxtaposition to the cortical body for several days penetrate between the cortical cords, and, the converse of the question, why do the sympathetic-chromaffine cells lie as a contiguous mass on the surface of the cortical body for several days before penetrating between the cords? To these questions we have no answer. The method by which the cells penetrate the gland may, however, be analyzed in this connection. Whitehead ('03), working on the pig, and Hett ('25), on the human embryo, state that the cells appear to follow a course of least resistance during

their movement into the gland, which Hett describes as amoeboid. If the movement, however, were entirely a random, amoeboid one, and if the individual cells followed the course of least resistance, the cells would not enter the gland at all, in the case of the chick, but would merely move around its sides, since the connective-tissue capsule does not inclose the gland till after the fifteenth day. Furthermore, the chromaffine cells lying at a distance from the cortical cords apparently show no migration or movement at this time. They continue to occupy the same relative position at the sides of the aorta and around the splanchnic plexus. Only the cells immediately around the cortical cords appear to be concerned in the penetration movement.

In this paper the penetration is described as a mass movement by the adjacent sympathetic-chromaffine cells, rather than as a movement of separate, individual cells. The complex as a whole shows successive stages of penetration. Many of the individual cells within the cell group are not oriented with their long axes parallel to the line of penetration. For this reason it is conceivable that the cortical cords may attract the adjacent cell complex. No suggestion can be made, however, as to the nature or mechanics of such an attraction. We do know that mass movements of cells occur, for example, in Triton embryos. The mechanics of these mass movements are likewise obscure at present.

SUMMARY

1. A study was made of the development of the suprarenal gland of the chick with reference to the following points: 1) Topographical relationship of the sympathetic cell masses to the cortical cords and to the sympathetic nervous system. 2) Cytological differentiation of the sympathetic cells.

2. The aggregation of indifferent sympathetic cells, although general over the entire medial surface of the cortical bodies, is more pronounced in the following regions: 1) Over the medial border of the anterior end. 2) In the middle of the gland, around mesonephric branches of the aorta, and on

the right side in the angle between the omphalomesenteric artery and the cortical body. 3) Along the medial surface of the posterior third of the gland, over the subcardinal veins, and over an anastomosis between them.

3. A dorsomedial and a ventromedial chain of ganglia develop on the surface of each gland. The location of the ganglia of each chain corresponds in position to the aggregation enumerated above. The indifferent cells between the aggregations penetrate the gland during differentiation of the medullary tissue.

4. Development in the gland during the sixth and seventh days is characterized by the differentiation of nervous tissue. The ganglia show marked development, the ganglionic cells developing from indifferent cells of the aggregations. Within the sympathetic cell masses many indifferent cells differentiate into nerve cells whose fibers innervate these rudiments. All cell groups of the rudiments are thus brought into association with each other.

5. At the same time, sympathetic cells which were inclosed between the cortical cords during the extension of the latter early on the fifth day, differentiate into nerve cells, whose fibers innervate the cortical body. By the seventh day, the fibers of these cells have also established association with those of the cell masses on the surface of the gland.

6. Differentiation of the 'chromaffine cells' begins early on the eighth day. During the process, cells which were formerly indifferent sympathetic cells show an accumulation of cytoplasmic granules, and an increasing tendency to show the 'chromaffine reaction.' The reaction becomes distinct in some cells at approximately 180 hours.

7. During differentiation, chromaffine cell groups having a direct nervous association with each other show identical stages of differentiation, as indicated by the intensity of the chrome reaction. No direct relationship is noted between intensity of reaction and proximity to the cortical cords.

8. Cytoplasmic inclusions, namely, Golgi apparatus and mitochondria in the chromaffine cells during differentiation, show little change in form from those of the indifferent cells.

9. Penetration of the chromaffine-medullary cells between the cortical cords is inaugurated at the same time that differentiation first appears. Except in early stages, penetration appears as a mass movement of cells rather than as an amoeboid movement of individual cells. The nerve cells of the cell masses are also carried into the gland by the movement.

10. In consideration of the intimate nervous associations between cell groups of the sympathetic-chromaffine complex at the time of differentiation of 'chromaffine cells,' and in view of the observation that directly associated cell groups show the reaction simultaneously and with equal intensity, it appears reasonable that differentiation (adrenaline secretion) may be directly dependent upon the sympathetic system. In view of the nervous associations between the cortical cords and the sympathetic-chromaffine complex, the fact that chromaffine-cell differentiation occurs in the general vicinity of the cortical body, and begins simultaneously with the penetration of the latter by the differentiating medullary cells adjacent to the cortical body, a dependence of this differentiation upon the cortical cells is not inconceivable.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

6 to 10 Differentiating nerve cells. Figures 6, 7, 8, cells from ganglia on surface of cortical body from seventh to twelfth days, showing Golgi material. Figures 9 and 10, multipolar cells lying between cortical cords prior to eighth day.

11 Sympathetic-chromaffine complex during its penetration between cortical cords on ninth day.

12 Sympathetic association between cell groups of sympathetic-chromaffine complex on eighth day. Differentiation of chromaffine cells.

13 and 14 Sympathetic associations with differentiating chromaffine cells on ninth day.

15 Golgi apparatus of indifferent cells, eighth day.

16 Golgi apparatus of differentiating chromaffine cells.

17 Various orientations of chromaffine cells lying between cortical cords on tenth day. Golgi apparatus taken as index of cell polarity.

18 and 19 Mitochondria of indifferent cells and of chromaffine cells, respectively.

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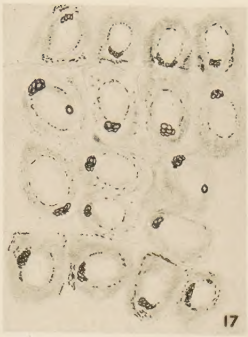
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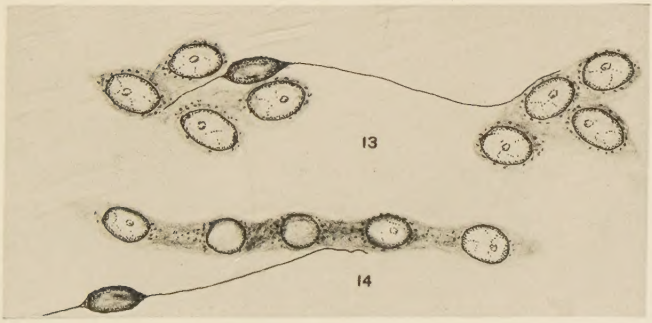
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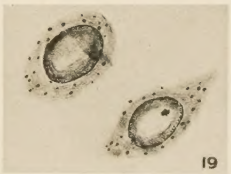
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